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ENTRAINMENT OF THE *NEUROSPORA* CIRCADIAN CLOCK

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Neurospora crassa has been systematically investigated for circadian entrainment behavior. Many aspects of synchronization can be investigated in this simple, cellular system, ranging from systematic entrainment and drivenness to masking. Clock gene expression during entrainment and entrainment without clock genes suggest that the known transcription/translation feedback loop is not alone responsible for entrainment in *Neurospora*.

Keywords *Neurospora*, Circadian rhythm, Entrainment, Clock, Oscillation

INTRODUCTION

The circadian clock is a self-sustained biological oscillator with a period of ~24 h in constant conditions. Sets of clock genes have been identified in animals, plants, fungi, and cyanobacteria, functioning as a transcription/translation negative feedback loop. Their mutation often results in a change in the free-running circadian rhythm. Circadian clocks in nature are, however, rarely subjected to the constant conditions that allow a free-running oscillation. They are normally exposed to a rhythmic environment, so that appropriate signals (zeitgebers), such as light, temperature, or occasionally even social cues, feed into the clock and entrain its oscillation to the 24 h day. The phase relationships of oscillating processes can be expected to be novel according to the entraining condition. They can move to different times of day or be suppressed depending on the structure of the cycle (Roden et al., 2002). Yet, entrainment is clearly the most relevant state for an organism's survival and also the state that was subjected to selection in the course of evolution (Roenneberg and Merrow, 2002a, 2002b). Because of the high complexity of circadian systems, both on the molecular-cellular as well as on the systemic level in higher plants and

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animals, we used a relatively simple model system, the filamentous fungus *Neurospora crassa* to study the molecular basis of circadian entrainment.

Neurospora is a haploid, filamentous fungus with a sequenced genome of only ~40 million base-pairs, annotated to about 10,000 genes (Galagan et al., 2003). A circadian rhythm in asexual spore (conidium) formation was first observed by Pittendrigh and coworkers (1959). In constant darkness, the period is about 22 h; in constant light, at any level greater than that approximating moonlight, conidia formation is arhythmic (Sargent et al., 1956). Conidiation was used to generate a panel of clock mutants, making *Neurospora* the second system for molecular approaches to circadian rhythms after *Drosophila* (Feldman and Hoyle, 1973; Konopka and Benzer, 1971).

The *Neurospora* clock gene *frequency* (*frq*) was cloned and used to demonstrate the concept of negative feedback in clock regulatory loops, by showing that FRQ-protein over-expression shuts down transcription from the endogenous *frq* locus (Aronson et al., 1994; McClung et al., 1989). Both the lack of functional *frq* RNA (Aronson et al., 1994a, 1994b; Loros and Feldman, 1986) and its constitutive over-expression result in arhythmicity. The activators of *frq* transcription include the blue light photoreceptor WC-1 and its partner WC-2 (Figure 1) (Ballario et al., 1996; Froehlich et al., 2002; He et al., 2002; Linden and Macino, 1997). The mechanism of negative feedback is likely not to act via direct interference with the transcription factor complex on the promoter. Rather, FRQ regulates the phosphorylation state of WC-1 and WC-2, which controls their activity (Schafmeier et al., 2005). This may also be the mechanism by which FRQ regulates WC-1 levels (Lee et al., 2000;

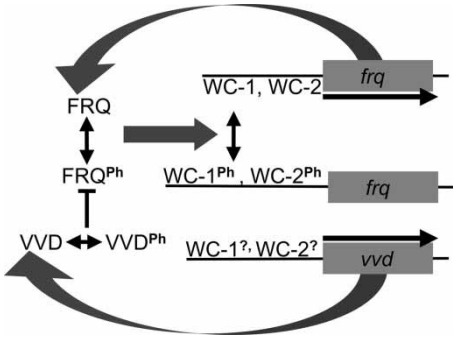

FIGURE 1 Molecular clock network in *Neurospora*. A transcription-translation negative feedback loop is described, whereby FRQ protein feeds back negatively on its own RNA expression. The transcriptional activators of *frq* and other light-induced genes include WC-1 and WC-2, whose activity is modified according to phosphorylation state, apparently the mechanism by which FRQ exerts negative feedback. In theory, it is possible the transcription factor complexes with mixed phosphorylation states could result in fine-tuning of transcriptional regulatory responses (shown here as WC-1$^{?}$ and WC-2$^{?}$ on the *vvd* promoter). The several kinases and phosphatases and post-transcriptional regulation that are part of clock regulation are omitted here.

Merrow et al., 2001), giving the appearance of both positive and negative feedback loops among one set of molecules. An additional photoreceptor, VIVID (VVD) is found in the cytoplasm (Schwerdtfeger and Linden, 2003), and it regulates the clock in entrainment, even though the free-running period of a *vvd* mutant is not different from that of a wild-type strain under numerous culture conditions (Elvin et al., 2005; Heintzen et al., 2001; Shrode et al., 2001).

We have set out to systematically describe the entrainment properties of *Neurospora* using many of the classical circadian protocols that have been used with other species for the purposes of comparison and validation of the *Neurospora* model system.

ENTRAINMENT WITH LIGHT

Entrainment is characterized by a stable phase reference point in relationship to an entraining cycle (*e.g.*, light/dark or warm/cold). A reference point or phase in the biological oscillation is chosen (*i.e.*, core body temperature nadir or melatonin onset in humans, or conidiation onset in *Neurospora*) and the difference between this internal time (phase) and a chosen reference of the external, zeitgeber cycle (lights on, lights off, midnight, *etc*.) is called the phase of entrainment. Phase can be expressed in real h or in degrees (with 360° representing a full zeitgeber cycle no matter how long or short its period is in real h). In the case of *Neurospora*, with the recent introduction of rigorous quantification methods (Roenneberg and Taylor, 2000), virtually any point in the conidiation cycle *can* be used as phase reference, allowing critical evaluation of the entire waveform, as it appears when entrained under different zeitgeber conditions. The waveform of the conidiation rhythm discussed here can be well described by using four standard phase reference points: onset, peak, offset, and trough. In the case of conidiation, onset (the upward transition through the non-rhythmic trend) has proven to be the most reliable marker for phase of entrainment (Roenneberg et al., 2005) so we will refer only to onset of conidiation here.

Entrained phase can be determined in 24 h cycles as well as in shorter or longer zeitgeber cycles (*e.g.*, T = 18 to T = 26 h). In non-24 h T-cycles, the biological clock is expected to entrain earlier in long cycles and later in short cycles, as shown decades ago, *e.g.*, with lizards and hamsters (Hoffmann, 1963; Pittendrigh and Daan, 1976). In symmetrical T-cycles with alternating light (L) and dark (D) conditions (LD, *e.g.*, 50% of each cycle in L and in D), *Neurospora* apparently breaks this rule in that conidiation onset lies ~7 h after dusk, irrespective of cycle length (Figure 2A) (Merrow et al., 1999). This finding suggested an hourglass system, with light driving the formation of the conidial band rather than it being controlled by an entrained biological oscillator—a puzzling result for a model circadian system.

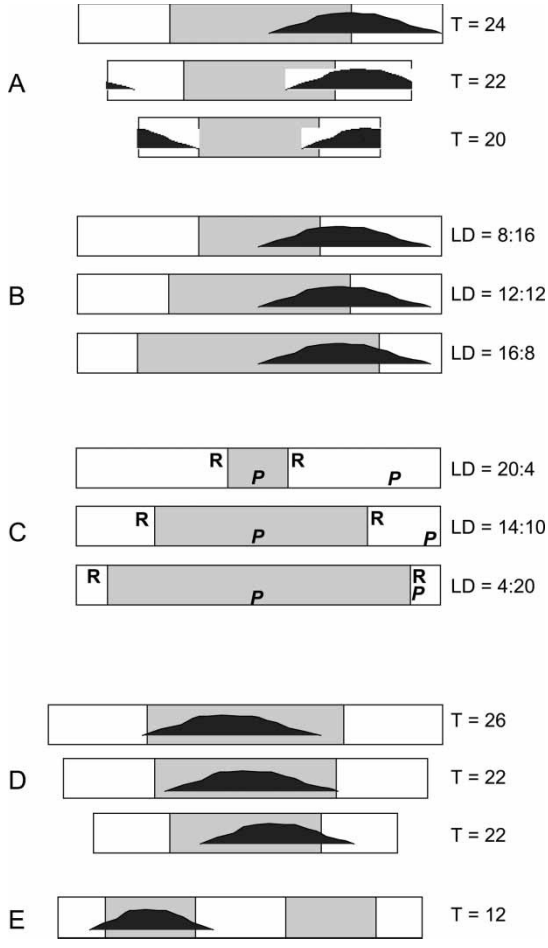

FIGURE 2 Summary of light and temperature entrainment protocols with the *band* strain of *Neurospora*. In panels A, B, and C, grey areas represent a dark incubation period; the open box is light. In panels D and E, the grey area represents cool temperature; whereas, the open area concerns the warm phase of the cycle. Panels A, B, D, and E use a cartoon of the conidial band to illustrate entrainment of banding. ***A***. T cycles with light show an onset of conidiation that occurs the same number of hours after darkness, independent of cycle length, thus showing driven, non-entrained states. ***B***. In cycles of different photoperiods but T = 24 h, conidiation onset is typically at midnight, indicating that both dawn and dusk signals are integrated for entrainment. ***C***. **R** represents when RNA is induced; ***P*** shows when protein is induced; a shadowed $\mathbb{R}$ and $\mathbb{P}$ indicate when RNA protein levels decrease, respectively. Although RNA levels respond predictably and directly according to light conditions, FRQ protein levels increase either rapidly or in an attenuated fashion when lights come on. The protein decreases at midnight, as conidiation is beginning. ***D***. Entrainment of conidiation in temperature cycles of different lengths is systematic, in that it is shows a later phase in short cycles, and an earlier one in long cycles. ***E***. *Neurospora* frequency demultiplies in T = 12 h temperature cycles.

We therefore probed the system with alternative entrainment protocols, *i.e.*, by systematically changing the duration of light (photoperiod) within a T = 24 h. When the phase of entrainment (judged by onset of conidiation) was measured in long and short photoperiods, it always appeared

around the middle of the night (Figure 2B; Tan et al., 2004a). Unlike a fixed relationship to dawn or dusk, a fixed relationship to midnight (independent of night length) means that the phase of entrainment, in reference to both dawn or dusk, changes systematically with photoperiod. Thus, the *Neurospora* clock shows entrainment under these conditions rather than drivenness (as described above).

We have additionally investigated molecular aspects of entrainment by light, focussing on FRQ for several reasons: induction of its RNA by light has been equated with phase resetting and entrainment (Collett et al., 2002; Crosthwaite et al., 1995; Elvin et al., 2005; Liu, 2003), and our work had shown that *frq* null mutants fail to entrain to light (Merrow et al., 1999). When *Neurospora* is transferred from darkness to light, both *frq* RNA and FRQ protein are rapidly induced (Collett et al., 2002; Crosthwaite et al., 1995, 1997). FRQ protein levels take 4 to 6 h until they reach a plateau in continuous light, while RNA levels are down-regulated following a peak at 30 min and are maintained at an adapted level, which is still higher than levels in dark-grown tissue. Similar experiments were performed with the reverse protocol, transferring *Neurospora* tissue from light to darkness, and these two single transition protocols were purported to explain entrainment (Collett et al., 2002; Liu, 2003). However, if *frq* or FRQ are involved in systematic circadian entrainment that occurs in T = 24 h photoperiod cycles, then they should be expressed with different kinetics in different cycles. We found this to be true for the protein but not the RNA (Figure 2C; Tan et al., 2004b). The *frq* RNA-kinetics are independent of photoperiod (and night length, scotoperiod). RNA is rapidly induced by the dawn light and photo-adapts during the day; it decreases rapidly after nightfall and then—but only in nights longer than 6 to 8 h—gradually increases during the scotoperiod. Thus, all aspects of RNA expression appear driven by non-circadian responses to the light environment. Because drivenness can be a special case of entrainment by a very strong signal that always resets the clock to a certain phase (Roenneberg et al., 2005), it would be more appropriate to state that *frq* RNA is masked by light (see below). The protein, however, is only induced rapidly (like it was in the single-release experiments) in short photoperiods. Its expression is substantially delayed in long photoperiods in spite of the rapid dawn-linked RNA-induction, indicating that post-transcriptional regulation of FRQ expression carries key information for circadian entrainment.

The systematic responses to different photoperiods on the molecular level in *Neurospora* invite the inquiry as to whether systematic entrainment according to season is meaningful in the *Neurospora* life cycle. We determined the quantitative yield of three light-regulated processes and found they were all specifically controlled by photoperiod (Tan et al., 2004a). Conidiation (asexual propagation) is most abundant in LD cycles of 12 : 12; more sexual spores are produced in 14 : 10 cycles; and mycelial

carotenoids increase over a broad range of photoperiods from about 10 to 20 h. In all cases, longer photoperiods decrease the output. In addition, these responses are disrupted in clock mutant strains, proving that these are not simply irradiance responses but represent photoperiodism in *Neurospora* that is somehow tied to the circadian clock.

To summarize, we can conclude the following about entrainment of the *Neurospora* clock by light and darkness: although it appears to be driven in symmetrical LD T-cycles, circadian entrainment is apparent when different photoperiods are used in the context of 24 h cycles. Systematic circadian entrainment can be seen at the level of FRQ protein, while *frq* RNA passively reacts to light (is masked), so that transcriptional regulation can be ruled out as a player of the entrainment mechanism. The benefit of entrainment for fitness exists both on the daily level (anticipation of changing environmental conditions) as well as on the seasonal level (*e.g.*, by the time-of-year-specific enhancement of reproduction).

ENTRAINMENT WITH TEMPERATURE

We investigated entrainment with another zeitgeber, namely temperature (Merrow et al., 1999). Although circadian systems are compensated for different temperature levels, they do respond to temperature changes; thus, clocks can also entrain to temperature as a zeitgeber. In symmetrical T-cycles, using alternations of 22 and 27°C, entrained phase is earlier in long and later in short cycles. Clock mutant strains with short or long free-running periods, respectively, also predictably and consistently entrain earlier or later (Figure 2D). When cycles are shortened to about half of the free-running rhythm (*e.g.*, T = 12 h for the wild type strain), a single conidiation bout occurs each 24 h (*i.e.*, every second cycle; a so-called 'frequency demultiplication'; Figure 2E). This demultiplication is an indication of a robust circadian oscillator.

Thus, the *Neurospora clock* performs as predicted for a (biological) oscillator in temperature cycles. Using onsets as phase reference points, it has been shown by a number of laboratories that even clock null strains (at the *frq* locus) show systematic entrainment in these T-cycles (Merrow et al., 1999; Pregueiro et al., 2005; Roenneberg et al., 2005) This is an exciting discovery, suggesting a multi-oscillator circadian system in *Neurospora*, like that seen in humans, rodents, flies, and plants (Aschoff et al., 1967; Grima et al., 2004; Honma et al., 1983; Johnson, 2001; Stoleru et al., 2004). Thus, the study of even a simple fungus (with neither organs nor a brain) shows the circadian system is a complex mechanism that consists of multiple oscillators, implying that this is an important adaptive feature of circadian clocks.

Experiments in constant conditions demonstrate that temperature regulates *frq* RNA splicing (Diernfellner et al., 2005). Thus, there are at least two levels—transcriptional, regulating the timing of RNA expression,

and post-transcriptional, regulating splicing—to control for different amounts of FRQ protein according to temperature signals (Liu et al., 1998). At the molecular level, temperature entrainment also contrasts light entrainment. Here, *frq* RNA is clearly *not* driven, appearing early in a long and later in a short cycle, and this indicates that it does not respond like an hourglass timer (Merrow et al., 1999).

MASKING

The behavior of *frq* RNA when light is used as a zeitgeber (Figure 2C) suggests 'masking' on the molecular level. Masking is an acute, non-circadian effect on the system that can, nonetheless, be induced by a zeitgeber signal. A common example in mice is that they typically stop running when lights are turned on, regardless of the time of day. This can confound straightforward determination of entrained phase, requiring release of the animals from the zeitgeber cycle into constant darkness, which allows an estimation of when they *would* have started running if their activity had not been acutely inhibited by light. Masking demonstrates that zeitgebers have effects other than dedicated circadian ones. So, although *frq* RNA is a component of the clock, it can be masked by light.

Conidiation in *Neurospora* can also show masking, as demonstrated in response to temperature (Pregueiro et al., 2005). Such effects are seen in the wild type strain, and can become even more prominent in the *frq*-less mutants (Merrow et al., 1999; Roenneberg et al., 2005). Changing zeitgeber strength is one of the best protocols to distinguish between entrainment (phase changes) and masking (phase remains the same) (Roenneberg et al., 2005).

DISCUSSION

The use of *Neurospora* to characterize entrainment principles has several advantages. To fully describe and eventually understand entrainment, a large number of experiments must be performed, which systematically scan different cycle lengths, amplitudes, and light or temperature portions (photo- and thermoperiod) of the zeitgeber in different clock mutants. These can be readily done in *Neurospora*, which is a powerful molecular genetic model system as well as an economical (non-animal) system.

Comparison of entrainment at the level of physiology and gene expression shows that regulation of *frq* RNA by the circadian system occurs in temperature but not in light cycles. Temperature cycles also demonstrate circadian clock characteristics (an entrainable, frequency de-multiplying oscillator) in *frq* null mutants, indicating that the exact function of the FRQ-WC transcription/translation feedback loop within the *Neurospora* clock must be reconsidered. Experiments (Merrow et al., 1999;

Merrow et al., 2001) and modelling (Roenneberg and Merrow, 1998, 1999, 2002a) indicate that input pathways into the circadian clock are both interfaces to the environment and integral components of the rhythm generating mechanism, so-called "zeitnehmers" (German for time taker, Roenneberg et al., 1998) that are, themselves, under circadian control. The frq/FRQ oscillator is part of the light input; when transducing light information, *frq* RNA is predominantly controlled by the input signal, while it shows its circadian regulation when the system is entrained *via* different inputs, *e.g.*, by temperature. Renewing the conceptual view of the FRQ-WC loop within the circadian system does not diminish its dominant role in clock function, *e.g.*, by determining chronotype and thereby phase relationships in general.

We used a mathematical model to simulate a circadian system that is composed of a network of feedback loops (Roenneberg and Merrow, 2002a). The individual feedbacks, when isolated, do not show circadian properties; however, the intact network does. Some could be involved in driving outputs, others in processing a specific zeitgeber (the zeitnehmer loops). A zeitnehmer feedback supplies rhythmic input, even in constant conditions (comparable to animals changing retinal light exposure by closing their eyes as part of the circadianly controlled behavior in constant light). Each of the feedbacks in the network is essential for the entire system, but experimental *in silico* mutagenesis suggests that they would be discovered more (close to or meditating zeitgeber input) or less often (distant from zeitgeber input) in mutant screens (Merrow and Roenneberg, 2005).

If we take *Neurospora* as a model system, then a logical extension is to ask: What is the zeitnehmer in other clock model systems? An intriguing example recently surfaced, with photoreceptor mutant mice (lacking melanopsin and rods) showing large changes in entrained phase, like *frq* mutants have shown (Mrosovsky and Hattar, 2005). So, at the level of the organism, photoreceptors engaged with the circadian system can regulate chronotype, a clear example of inputs determining phase. The mammalian circadian system is hierarchical in the sense that many cells have been demonstrated to oscillate as cellular clocks, even when cultured as single-cell suspensions (Balsalobre et al., 1998; Welsh et al., 1995). If they are each a clock, then a constructive approach is to ask: Which cellular clock components serve as zeitnehmers? In some cases, what we think of as central clock genes may be functioning in this role.

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